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Plasma Delivery of Retinoic Acid to Tissues in the Rat*

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All-*trans*-retinoic acid (RA) activates ligand-dependent transcription factors that regulate retinoid-responsive gene expression. It is assumed that all-*trans*-RA is formed within cells through *in situ* oxidation of retinol derived from the circulation. However, the circulation contains low levels of all-*trans*-RA (approximately 0.2–0.7% of that of plasma retinol). Our studies investigated the extent to which plasma all-*trans*-RA contributes to tissue pools of this retinoid and explored factors responsible for regulating its uptake by tissues and cells. Rats were continuously infused, to steady state, with all-*trans*-[^{3}H]RA. From measures of specific activities of all-*trans*-[^{3}H]RA at steady state, we determined that the preponderance of all-*trans*-RA in brain and liver was derived from the circulation. For six other tissues, approximately 10–30% of the retinoid was derived from the circulation, but pancreas and testis derived very little from the circulating pool. In other studies, we showed that retinoid nutritional status influences clearance of a bolus dose of all-*trans*-RA and that neither the rate of cellular all-*trans*-RA uptake nor its intracellular half-life is influenced by cellular lipid levels. Taken together, our data indicate that plasma all-*trans*-RA contributes to tissue pools of this retinoid and that specific and physiologically responsive cellular processes mediate its uptake.

Retinoids (vitamin A) are essential for vision, reproduction, growth and differentiation, and maintenance of the general health of the organism (1–3). Retinoic acid and possibly some of its metabolites are the active retinoid forms responsible for mediating most of the nonvisual functions of this class of compounds. Both 9-*cis*- and all-*trans*-retinoic acid interact in the nucleus with ligand-dependent transcription factors to regulate expression of retinoid-responsive genes (4). Two distinct classes of nuclear receptors for retinoic acid have been described; these include the retinoic acid receptors (RARα, -β, -γ)[1] and the retinoid X receptors (RXRα, -β, -γ) (4). Response elements for these transcription factors have been characterized for a diverse group of genes including those for laminin B1, RARβ, cellular retinol-binding protein, type I (CRBP), apolipoprotein A-I, oxytocin, and phosphoenolpyruvate carboxykinase (3, 4). Review of the literature indicates that retinoids mediate expression of over 150 genes (5).

Ultimately, all retinoids in the body are derived from the diet. The bulk of dietary retinoid, consumed as either preformed vitamin A or provitamin A (carotenoids), is taken up in the intestine and packaged as retinyl ester, along with other dietary lipids, in chylomicrons (6–8). The majority of this retinyl ester is delivered to the liver (6–8). A small portion of the dietary retinyl ester in chylomicrons is taken up by some extrahepatic tissues, particularly bone marrow, kidney, and adipose tissue, where the retinyl ester may serve as a source of retinoid for meeting tissue needs (7). Within the liver, the newly arrived dietary retinoid is either stored as retinyl ester in hepatic stellate cells or secreted into the circulation as retinol bound to its specific plasma transport protein, retinol-binding protein (RBP) (7, 9). It is generally thought that tissue retinoid needs are satisfied primarily through the delivery of retinol to cells via RBP. Plasma retinol is internalized by cells from RBP through a process involving the action of CRBP (9, 10). Within the cell, the retinol can be oxidized to retinoic acid through enzymatic processes (7) which have also been proposed to involve CRBP (11, 12).

In addition to the transport process described above, other pathways for the delivery of retinoid to tissues may operate within the body. A small fraction of dietary retinoid is converted to retinoic acid in the intestine (or may arrive as such in the diet) and is absorbed via the portal system as retinoic acid bound to albumin (7, 8, 12). In plasma, retinoic acid circulates bound to albumin (12). The fasting plasma level of all-*trans*-retinoic acid is very low, being in the range of 4–14 nmol/liter in humans (about 0.2–0.7% of plasma retinol levels) (13, 14) and 7.3–9 nmol/liter in rats (15, 16). It is presently not clear to what extent plasma retinoic acid contributes to tissue pools of this retinoid. Our studies reported below address this question. In addition, they explore whether retinoic acid accumulation by tissues from plasma is specific and regulated in response to physiologic state and whether the lipid content of a cell influences either the rate of uptake or half-life of retinoic acid within a cell.

EXPERIMENTAL PROCEDURES

Animals and Surgical Procedures—Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) ranging in weight from 400 to 500 g and maintained on a commercial chow diet (Ralston Purina Inc., St. Louis, MO), without any source of carotenoid, were employed for all of our studies. Rats were anesthetized with a mixture of ketamine and xylazine prior to jugular vein cannulation. Polyethylene catheters were inserted into both the right and left jugular veins and externalized by routing through subcutaneous dorsal tunnels (17). The catheters were fixed with dental cement to the cranial area, and the lines were filled with either 200 units of sodium heparin in saline or a mixture of 50 units of sodium heparin and 40% polyvinylpyrrolidone in saline to ensure that they remained patent. The catheterized rats awoke within 1–3 h after surgery and were allowed to recover for at least 1 day prior to the start of the experiments. Animals were allowed

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[1] The abbreviations use are: RAR, retinoic acid receptor; RXR, retinoid X receptor; HPLC, high performance liquid chromatography; PBS, phosphate-buffered saline; RBP, retinol-binding protein; CRBP, cellular retinol-binding protein, type I; CRABP, cellular retinoic acid-binding protein, type I; FCR, fractional catabolic rate; ACR, absolute catabolic rate.

free access to water and chow throughout the studies.

Continuous and Bolus Infusion Studies—To study the contribution of plasma retinoic acid to tissue retinoic acid pools, physiologic doses of all-*trans*-[^{3}H]retinoic acid (50.6 Ci/mmol, DuPont NEN) were infused continuously into control animals. The retinoic acid dose consisted of 1–1.5 μCi of all-*trans*-[^{3}H]retinoic acid dispersed in 1.0 ml of 1% fatty acid-free rat albumin (the physiologic carrier of retinoic acid in the circulation) (12) in phosphate-buffered saline (PBS; 10 mM sodium phosphate, 150 mM sodium chloride, pH 7.2). The infusate was pumped through one jugular cannula at a flow rate of 0.78 ml/h. The infusion rate was chosen, based on preliminary studies, to provide measurable radioactivity in tissues without significantly perturbing the plasma retinoic acid pool. Three to five serial blood samples (each of approximately 0.5 ml) were taken from the second jugular cannula at 1-h intervals to establish that the plasma all-*trans*-[^{3}H]retinoic acid levels had reached a steady state. Immediately following the final sampling and usually 5 h after the start of infusion, the animals were sacrificed with a mixture of ketamine and xylazine, and tissues (brain, liver, kidney, epididymal fat, perinephric fat, seminal vesicle, epididymis, pancreas, and testis) were quickly perfused with ice-cold PBS and excised for HPLC analysis of retinoic acid levels. Urine was collected throughout the infusion period so that the hydration status of the animal could be monitored.

For some studies, rats were given a bolus injection of a dose consisting of 5 μCi of all-*trans*-[^{3}H]retinoic acid (50.6 Ci/mmol) and 0.5 μCi of [^{14}C]oleic acid (55.5 mCi/mmol) in 1.0 ml of 1% fatty acid-free rat albumin in PBS through one jugular cannula. Between 1.5 and 10 min after injection, three to five serial blood samples (each of approximately 0.5 ml) were taken from the second jugular cannula for analysis of [^{3}H]retinoic acid and [^{14}C]oleic acid remaining in the circulation. At 10 min, the injected animals were anesthetized with ketamine and xylazine; the whole body was perfused with ice-cold PBS; and brain, liver, kidney, epididymal fat, perinephric fat, seminal vesicles, epididymis, pancreas, and testis were taken for extraction and analysis of tissue levels of [^{3}H]retinoic acid and [^{14}C]oleic acid as described in the section below.

In preliminary experiments developing and characterizing the methodologies used for our studies, it quickly became obvious that plasma retinoic acid was being taken up by most tissues and was very rapidly being metabolized. We observed that after the stoppage of the circulation of a perfused rat (*i.e.* upon sacrifice) the metabolism of retinoic acid by tissues continued. Thus, a critical point in the design of these experiments was to allow very little time to elapse between the stoppage of circulation and the dissection of tissues. This ensured that our measures provided accurate estimations of the contribution which plasma retinoic acid makes to tissue pools. With this in mind and as part of our experimental design, we limited the maximum period of time between stoppage of the circulation and flash freezing of the final dissected tissue to 5 min. Only approximately 10 tissues could be dissected and processed (weighed and frozen) in 5 min. Because of this experimental limitation, we chose to study only the 10 tissues listed above.

Extraction of Retinoic Acid from Plasma and Tissues—All extraction and analytical procedures were carried out in a darkened room using brown glass tubes to protect the retinoids from exposure to light. Plasma samples were diluted with equal volumes of PBS prior to extraction. Tissues were homogenized in PBS (2 ml of PBS/g of tissue) using three 15-s pulses of a Brinkmann Polytron PT 300 homogenizer (Brinkmann Instruments), at setting 5 on the homogenizer. An internal standard consisting of a known amount of all-*trans*-7-(1,1,3,3,-tetramethyl-5-indanyl)-3-methyl-octa-2,4,6-trienoic acid (kindly provided by Dr. A. Levin, Hoffmann-LaRoche, Inc., Nutley NJ) was added in 0.1 ml of ethanol to each plasma or tissue sample in order to monitor the recovery of retinoic acid during the extraction and HPLC procedures (18). Retinoic acid was extracted from the tissue homogenates and plasma using a modification of the procedure described by Tang and Russell (19, 20). This extraction procedure is quite gentle and does not cause retinoyl-β-glucuronide hydrolysis. Briefly, plasma and tissue homogenates (3 ml) were extracted twice with chloroform/methanol (2:1), and the chloroform extracts were combined and concentrated, under a gentle stream of N_2, to a final volume of less than 1 ml. This retinoid-containing chloroform extract was then applied to 100 or 500 mg aminopropyl solid phase extraction columns (Baxter Labs. Inc., Chicago) that had previously been equilibrated with hexane. Under these chromatographic conditions, most lipids are retained by the column. The neutral lipids were eluted first from the column with 5 ml of chloroform/isopropanol (2:1). After the neutral lipids were eluted, the retinoic acid was eluted from the aminopropyl column with 5 ml of 2% acetic acid in diethyl ether containing 0.01% butylated hydroxytoulene as an antioxidant. The acetic acid/diethyl ether eluates were collected, evaporated to dryness under a gentle stream of N_2, and redissolved in HPLC mobile phase (hexane/acetonitrile/acetic acid, 99.5:0.4:0.1) for injection onto the HPLC.

HPLC Analysis—All-*trans*-retinoic acid levels were determined by normal phase HPLC employing two silica columns linked in tandem. The silica columns consisted of a 3.9 × 150-mm Waters 5 μ Resolve (Waters Associates, Milford, MA) followed by a 4.6 × 150-mm 3 μ Supelcosil LC-Si (Supelco Inc., Bellefonte, PA). The first column was preceded by a Waters silica Guard-PAK guard column. For chromatography, we employed an isocratic system where the mobile phase consisted of hexane/acetonitrile/acetic acid (99.5:0.4:0.1) flowing at 1.8 ml/min. The mobile phase was made fresh daily and filtered and degassed immediately prior to use. The solvent was delivered by a Varian Star 9010 pump (Varian Instruments, Sugarland, TX). We routinely injected 90 μl of sample onto the columns using a Varian Star 9095 Autosampler. Retinoic acid mass was detected at 350 nm using a Spectra-Physics Spectra 100 variable wavelength detector (Spectra Physics Inc., Piscataway, NJ). All-*trans*-retinoic acid levels were quantitated from the integrated area under its peak using a standard curve, constructed with authentic standards of all-*trans*-retinoic acid of known mass. All-*trans*-[^{3}H]retinoic acid was detected and quantitated with an in-line Berthold LB506C-1 radioactivity monitor (EG&G Berthold, Nashua, NH). This normal phase system was selected because it allowed for maximal detection of ^{3}H counts/min compared to other normal phase and reverse phase HPLC systems, where more quenching of radioactivity was observed.

For standards, authentic all-*trans*- and 9-*cis*-retinoic acid were obtained as a kind gift of Dr. Art Levin (Hoffmann-La Roche, Inc., Nutley, NJ) and authentic 13-*cis*-retinoic acid was obtained from Sigma. Low limits of detections for all-*trans*-, 13-*cis*-, and 9-*cis*-retinoic acid in our HPLC assay were estimated to be respectively, 0.7, 1.0, and 0.7 ng/g tissue.

Nutritional Studies—For some studies, rats were maintained for 10 weeks on one of two different nutritionally complete purified diets which supplied different levels of retinoid. The purified diets assessed were a nutritionally complete control diet which contained 2.4 μg of retinol/g of diet and a totally retinoid-deficient but otherwise complete diet. These diets and their use have been described in detail in the literature (21, 22). After 10 weeks on the diets, bolus infusion studies were carried out on these animals as described above.

Adipocyte Cell Cultures—To determine whether lipid content of cells and tissues influences retinoic acid uptake and retention we investigated the ability of cultured preadipocytes and adipocytes to take up all-*trans*-retinoic acid from the culture medium. BFC-1β preadipocytes and adipocytes were cultured and maintained according to procedures we have previously described (23–25). Preadipocytes were cultured in 75-cm^2 plastic tissue culture flasks. For our studies of retinoic acid uptake and metabolism, the preadipocytes were used on the day they reached confluence. Similarly, BFC-1β adipocytes were cultured in 75-cm^2 flasks and were used when greater than 75% of the cells present in the flask had undergone adipose conversion as evidenced by the presence of characteristic lipid droplets within the cells. Five μCi of all-*trans*-[^{3}H]retinoic acid in 5 μl of ethanol were dispersed in 1.0 ml of fatty acid-free rat albumin in PBS, and 0.1 ml of the dispersion was added directly to 3 ml of the appropriate culture medium (23–25). The final concentration of the all-*trans*-retinoic acid in the culture medium was 3×10^{-8} M. After the labeled medium was provided to the cells, they were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for periods of up to 6 h. After incubation, the medium was removed for analysis of retinoic acid. The cells were washed three times with ice-cold PBS and scraped from the plate into a small volume of PBS. The medium and cells were immediately frozen in liquid N_2 and stored at −70 °C for up to 1 week prior to analysis for retinoic acid levels. Retinoic acid was extracted from the cells and medium and analyzed as described in the above sections. The half-life of all-*trans*-retinoic acid in preadipocytes and adipocytes was calculated from the slope obtained by regression analysis of a linear plot of total system (cells + medium) all-*trans*-retinoic acid levels and time (for times ranging from 1 through 6 h after addition of all-*trans*-retinoic acid to the medium). For these plots, at each time of analysis, cellular and medium all-*trans*-retinoic acid levels were determined in triplicate, and the average value was used for half-life calculation.

Mathematical Modeling and Statistical Methods—The specific activity of [^{3}H]retinoic acid present at steady state in each tissue (and plasma) was determined from individual measurements of radiolabeled retinoic acid (dpm/g of tissue) and retinoic acid mass (pmol or ng/g of

tissue) carried out for each tissue. The purpose of the continuous infusion of all-*trans*-[^{3}H]retinoic acid was to establish a steady state among the infused retinoic acid, plasma retinoic acid, and tissue retinoic acid pools. Since the pool of retinoic acid present within each tissue must be composed of the retinoic acid synthesized endogenously by the tissue from retinol oxidation (and possibly from retinoyl-β-glucuronide hydrolysis (26) but not carotenoid cleavage, because the rats employed for our studies received no carotenoid in the diet) and the retinoic acid taken up from the circulation, the contribution that plasma retinoic acid makes to a tissue pool is simply the ratio of the specific activity of all-*trans*-[^{3}H]retinoic acid present in the tissue to plasma all-*trans*-[^{3}H]retinoic acid specific activity:

$$\%\ \text{Plasma contribution} = \frac{\text{Tissue retinoic acid specific activity}}{\text{Plasma retinoic acid specific activity}} \times 100\% \quad \text{(Eq. 1)}$$

The fractional catabolic rate (FCR) in plasma pools per hour was determined from the following equation:

$$\text{FCR} = \frac{[^3\text{H}]\text{Retinoic acid infusion rate in dpm/h}}{(\text{dpm } [^3\text{H}]\text{Retinoic acid/ml plasma}) \times W \times 0.04} \quad \text{(Eq. 2)}$$

where W is the weight of the rat in grams and rat plasma volume is assigned to be 0.04 ml/g total body weight (27). The absolute catabolic rate (ACR) in ng/h was determined from the following equation:

$$\text{ACR} = \frac{[^3\text{H}]\text{Retinoic acid infusion rate in dpm/h}}{\text{Plasma retinoic acid specific activity in dpm/ng}} \quad \text{(Eq. 3)}$$

Since we were concerned that residual plasma contamination of our perfused tissues might influence our calculations, we determined the amount of plasma contamination in the perfused tissues of four representative rats (which were different from those employed for retinoic acid continuous infusion). For this purpose, a bolus dose of ^{125}I-RBP, prepared as described previously (28), was intravenously injected in PBS into the rats through a jugular vein catheter and allowed to circulate for 3–5 min. This time period is sufficiently short so that very little ^{125}I-RBP would be expected to be taken up by cells within the rat tissues (29). The animals were then sacrificed, and tissues were perfused and excised as described above for the experimental animals. Levels of ^{125}I in the tissues and plasma were measured directly using an LKB RIAGAMMA (LKB-Pharmacia Instruments, Piscataway, NJ). The maximum residual plasma contamination of each tissue was calculated assuming that the level of ^{125}I measured in each tissue arose solely from the residual plasma present in the tissue. From these measurements, we determined that the residual plasma contamination for each tissue was both very small (<1% of the total ^{125}I counts/min present in the circulation) and very reproducible for the four rats. Our calculations of the percent contribution which plasma retinoic acid makes to tissue pools were made both uncorrected and corrected for possible residual plasma content of the tissues. The two sets of calculated values were found to be very similar, and for no tissue were differences of greater than 5% observed. The data in this report are given without correcting for average residual plasma present in each tissue.

For the bolus studies, only three to five samples could be obtained from each animal. Therefore, the data from all animals on the same diet had to be pooled. With this in mind, different sampling times were used for different animals so that, taken together, data were available at seven to eight distinct time points for each diet. The data were expressed as percent of injected dose and were fitted by a two-pool model to estimate plasma FCR (30). The tissue data were expressed as percent of injected dose per g of tissue at 10 min after injection. Retinoic acid and oleic acid levels were compared by paired t tests.

RESULTS

Steady State—In preliminary experiments with approximately 20 rats, we demonstrated that, for our experimental conditions, a steady state in plasma retinoic acid and tissue retinoic acid pools was established within 2.5 h after the start of continuous retinoic acid infusion. Repeated plasma samples (usually three or four) were taken over 6 h (for some animals this period ranged up to 24 h) after the start of infusion and plasma all-*trans*-[^{3}H]retinoic acid specific activity was measured. When computer-determined slopes of plots of plasma retinoic acid specific activities for different animals were statistically analyzed, they were not found to be significantly different from zero, the slope of a horizontal line. Thus, when plasma all-*trans*-[^{3}H]retinoic acid specific activity was plotted as a function of time, for times greater than 2.5 h, there was no consistent trend with time. We concluded from this analysis that a steady state was reached within 2.5 h after the start of infusion. This conclusion was also supported by measures of ^{3}H counts/min levels in urine collected over defined time intervals which began 2.5 h after infusion started. Although none of the ^{3}H counts/min present in the urine were present as retinoic acid, the total ^{3}H counts/min determined in the timed urine samples were reproducibly found to be equal to the total all-*trans*-[^{3}H]retinoic acid infused into the animal over the time interval of urine collection. The observation that the radioactivity infused into an experimental animal equals that being excreted by the animal would indicate that the whole body pool of retinoic acid is at isotopic steady state. These two observations, when taken together, fully support the conclusion that plasma and tissue retinoic acid pools are in a steady state condition within 2.5 h after the start of all-*trans*-[^{3}H]retinoic acid infusion. This conclusion is further supported by our data which indicate that the fractional catabolic rate of plasma all-*trans*-retinoic acid is very large (see below).

Tissue Distribution—We employed sensitive analytical procedures which enabled us to measure simultaneously all-*trans*-retinoic acid mass and radioactivity in all tissues examined. To obtain the simultaneous profiles, a UV absorbance detector was linked in series with an in-line radiation monitor. The extraction procedure did not hydrolyze retinoyl-β-glucuronides to retinoic acid (19, 20). Levels were measured in the brain, liver, kidney, epididymal and perinephric fat, seminal vesicle, spleen, epididymis, pancreas, testis, and plasma for eight rats which were continuously infused for 5–6 h with all-*trans*-[^{3}H]retinoic acid. Representative HPLC profiles for the simultaneous determination of retinoic acid mass (UV absorbance) and radioactivity for extracts prepared from liver, brain, epididymal fat, and testis of one experimental animal are shown in Fig. 1. The upper portion of each profile gives the UV absorbance and the lower portion ^{3}H counts/min. As can be seen from the markers in Fig. 1, the normal phase HPLC procedure we employed was able to resolve 13-*cis*-, 9-*cis*-, and all-*trans*-retinoic acid. In our system, pure authentic all-*trans*-didehydroretinoic acid eluted approximately 2 min after all-*trans*-retinoic acid.

The tissue levels of all-*trans*-retinoic acid are given in Table I. The tissue with the highest mean level of all-*trans*-retinoic acid was the pancreas, and the lowest mean level was observed in the brain. All-*trans*-retinoic acid was present in all tissues examined for each of the eight rats; however, as seen in Fig. 1, only trace amounts of 13-*cis*- or 9-*cis*-retinoic acid were detectable in any tissue extract. Although we did not specifically plan to determine the levels of these *cis* isomers in tissues, we can see no reason why our extraction and HPLC procedures would selectively allow determination of all-*trans*-retinoic acid and not of the two *cis* isomers. This suggests to us that 13-*cis*- and 9-*cis*-retinoic acid were present in the tissues examined at very low levels relative to those of all-*trans*-retinoic acid and that there was little or no isomerization of the infused all-*trans*-[^{3}H]retinoic acid.

From serial plasma samples and measurement of plasma all-*trans*-[^{3}H]retinoic acid specific activity, we established that a steady state was reached in each of the eight experimental animals. At steady state, the extent to which plasma contributes to the retinoic acid pool within a tissue is simply the ratio of tissue retinoic acid specific activity to plasma specific activity (Equation 1). Table II gives the plasma retinoic acid contribution to retinoic acid pools in the 10 tissues investigated. The

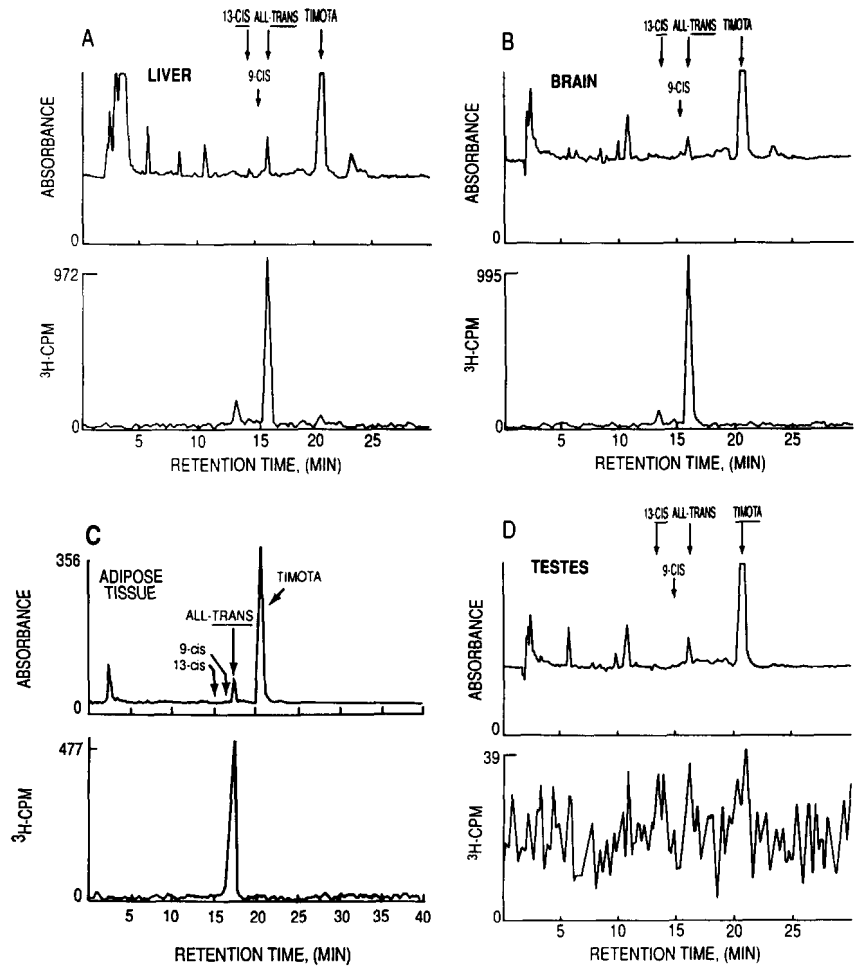

FIG. 1. **Representative normal phase HPLC profiles for retinoic acid from tissue extracts prepared from the same rat for liver (*panel A*), brain (*panel B*), adipose tissue (epididymal fat) (*panel C*), and testis (*panel D*).** The preparation of the extracts and the HPLC procedures were carried out as described under "Experimental Procedures." The *upper portion* of each panel gives the profile detected by measurement of UV absorbance at 350 nm and the *lower portion* the profile of ^{3}H counts/min.

TABLE I
Tissue concentrations of all-trans-retinoic acid

Tissue	*n*	All-*trans*-retinoic acid	
		ng/g tissue	*pmol/g tissue*
Brain	8	1.9 ± 1.0[a]	6.3 ± 3.3
Liver	7	3.4 ± 1.4	11.3 ± 4.7
Kidney	8	2.5 ± 1.2	8.3 ± 4.0
Epididymal fat	8	4.7 ± 3.7	15.7 ± 12.3
Perinephric fat	8	3.8 ± 2.6	12.7 ± 8.7
Seminal vesicle	8	3.6 ± 2.1	12.0 ± 7.0
Spleen	5	3.8 ± 3.6	12.7 ± 12.0
Epididymis	8	4.2 ± 1.6	14.0 ± 5.3
Pancreas	6	8.8 ± 4.9	29.3 ± 16.3
Testis	8	3.2 ± 0.8	10.7 ± 2.7
Plasma	8	0.5 ± 0.2[b]	1.8 ± 0.7

[a] All values are given as mean ± S.D.
[b] The concentration of all-*trans*-retinoic acid in the plasma is given in terms of ng/ml or pmol/ml of plasma.

TABLE II
Contribution of plasma retinoic acid to tissue retinoic acid pools

Tissue	*n*	Contribution
		%
Brain	8	88.4 ± 21.9[a]
Liver	8	78.2 ± 28.2
Kidney	8	33.4 ± 17.1
Epididymal fat	8	30.2 ± 29.9
Perinephric fat	8	24.5 ± 14.5
Seminal vesicle	8	23.1 ± 13.5
Spleen	5	19.0 ± 10.1
Epididymis	8	9.6 ± 6.1
Pancreas	6	2.3 ± 2.5
Testis	8	0.7 ± 0.9

[a] All values are given as mean ± S.D.

values presented in Table II indicate that retinoic acid pools in two tissues (brain and liver) are derived almost entirely from the plasma; two other tissues, pancreas and testis, derive almost no retinoic acid from the circulation. Other tissues (kidney, epididymal fat, perinephric fat, seminal vesicle, spleen, and epididymis) derive a minority of their retinoic acid pools from the circulation. It should be noted that the mathematical model used for calculating these percent contribution values assumes that tissue all-*trans*-retinoic acid pools are kinetically homogeneous. We are not aware of any data which contradict the validity of this assumption, nor do any of our data suggest that this is not the case.

From our data obtained with continuously infused animals, it was also possible to calculate the FCR and ACR for all-*trans*-retinoic acid in our experimental animals. These are provided in Table III. These values are consistent with the conclusion that all-*trans*-retinoic acid is rapidly turning over in the rat. The FCR indicates that the plasma pool of all-*trans*-retinoic acid is turned over approximately every 2 min.

Bolus Studies for Different Diets—Since the FCR indicates that the turnover of plasma retinoic acid is very rapid, we also carried out studies to assess the rate of plasma clearance of all-*trans*-[^{3}H]retinoic acid given as a bolus dose. As part of these studies, we asked both whether plasma free fatty acids (which like retinoic acid circulate bound to albumin) are cleared from the plasma at the same rate as retinoic acid and whether retinoid nutritional status influences plasma clearance of all-*trans*-retinoic acid and/or free fatty acids. For this purpose, a bolus dose consisting of tracer amounts of all-*trans*-[^{3}H]retinoic acid and [^{14}C]oleic acid bound to rat albumin was

TABLE III
Fractional and absolute catabolic rates of retinoic acid in control rats

	n	Rate[a]
Fractional catabolic rate	7	30.4 ± 12.0 h^{-1}
Absolute catabolic rate	8	640 ± 260 pmol/h
		192 ± 78 ng/h

[a] All values are given as mean ± S.D.

injected intravenously into rats maintained on one of two purified diets which differed only in retinoid content. The diets consisted of a nutritionally complete control diet providing 2.4 μg of retinol/g of diet and a totally retinoid-deficient diet but otherwise nutritionally complete diet. The animals receiving the totally retinoid-deficient diet, at the time of their use for these studies, all had plasma retinol levels ranging between 1.8 and 4.2 μg/dl compared to a range of 21.4–26.3 μg/dl for the control fed animals. The results of these bolus studies are presented in Fig. 2 along with fitted curves. The calculated FCRs are given in Table IV. As can be seen from Fig. 2 and Table IV, plasma retinoic acid is cleared rapidly from the circulation but at a slower rate than oleic acid. Also, in total retinoid deficiency, the rate of clearance of plasma all-*trans*-retinoic acid is increased significantly but the rate of oleic acid clearance remains unchanged. The FCR estimated from the bolus data for the control-fed group is approximately 41% of that estimated from the continuous infusion studies of chow-fed rats. Although the two FCR values for all-*trans*-retinoic acid estimated using these two independent approaches are not in very close agreement, they do both indicate that all-*trans*-retinoic acid is being rapidly turned over in the circulation. Similar rates for plasma clearance of all-*trans*-retinoic acid have also been observed when large pharmacologic doses were injected intravenously into rats (31, 32). The rate of clearance of plasma oleic acid that we observed in our studies is very similar to the rate reported for the physiologic clearance of fatty acids from the circulation of rats (33).

The animals receiving the bolus doses were sacrificed after 10 min, and levels of ^{3}H and ^{14}C counts/min in total lipid extracts prepared from tissues were assessed. The distribution of ^{3}H and ^{14}C counts/min in tissues is given in Table V. These tissues differentially took up retinoic acid and oleic acid. As would be expected, the brain took up very little oleic acid from the circulation; but as can be seen in Table V, retinoic acid uptake was substantial in the brain. On the contrary, the liver took up relatively high levels of both retinoic acid and oleic acid. Brain, seminal vesicle, epididymis, and testis, regardless of dietary group, took up significantly more ($p < 0.05$) of the dose of retinoic acid than of the oleic acid dose. Liver, kidney, epididymis, and pancreas from retinoid-deficient animals were found to take up significantly more ($p < 0.05$) retinoic acid than corresponding tissues from animals fed the control diet. The observation that tissues differentially take up all-*trans*-retinoic acid given in a bolus trace dose validates our finding in Table II that plasma all-*trans*-retinoic acid contributes to tissue pools in a tissue dependent manner.

Cell Studies—To gain additional insight into the cellular processes and mechanisms responsible for retinoic acid uptake and clearance, we carried out studies which asked both if cells of common origin take up and metabolize retinoic acid at similar rates and if cellular lipid content influences the uptake and accumulation of the fat soluble retinoic acid. Since the BFC-1β preadipocytes can be induced to differentiate into lipid-laden adipocytes upon addition of insulin and thyroid hormone to the culture medium (24, 25), we investigated all-*trans*-[^{3}H]retinoic acid uptake and its rate of metabolism in both BFC-1β preadipocytes and adipocytes. As seen in Fig. 3, all-*trans*-[^{3}H]retinoic

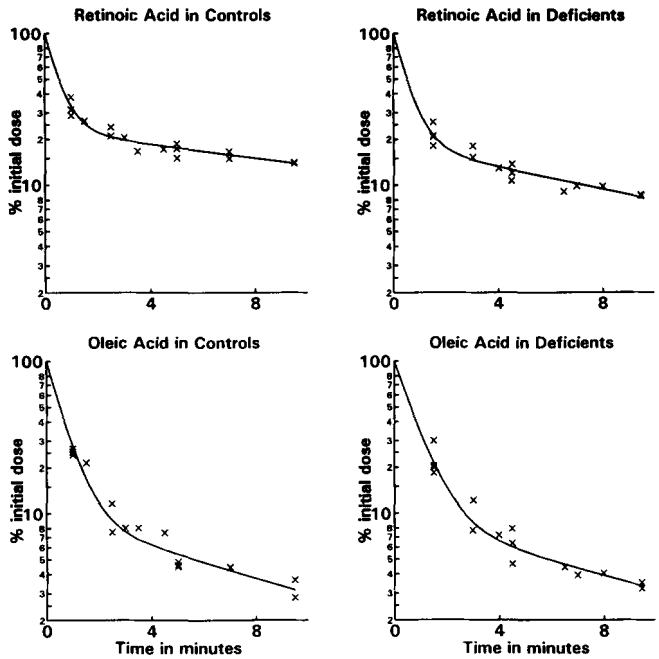

FIG. 2. **Plasma clearance curves for bolus doses of all-*trans*-[^{3}H]retinoic acid (*upper panels*) and [^{14}C]oleic acid (*lower panels*) given simultaneously to rats maintained on a control (*left panels*) or a totally retinoid-deficient (*right panels*) diet.** For this figure, curves were fitted starting at 100% at zero time. Individual measures of all-*trans*-[^{3}H]retinoic acid or [^{14}C]oleic acid from which the curves were fitted are indicated (×) in each panel. All procedures were carried out as described under "Experimental Procedures."

TABLE IV
Effect of diet on fractional catabolic rates of all-trans-[^{3}H]retinoic acid and [^{14}C]oleic acid given by simultaneous intravenous bolus dose

Diet	Fractional catabolic rate[a]	
	All-*trans*-retinoic acid	Oleic acid
Control	12.5 ± 4.6[b]	42.9 ± 6.9[c]
Deficient	22.7 ± 5.5[d]	38.1 ± 7.2[c]

[a] Values for the fractional catabolic rates are given in terms of plasma pools per h ± 95% confidence intervals.
[b,c,d] Differently superscripted values are significantly different ($p < 0.05$) from each other.

acid is rapidly taken up from the culture medium by both BFC-1β preadipocytes and adipocytes. Levels of all-*trans*-[^{3}H]retinoic acid within the preadipocytes and adipocytes reach a maximum within 30 min after addition of all-*trans*-[^{3}H]retinoic acid to the medium. Thus, it would appear that neither the amount nor the rate of uptake of retinoic acid by cells is markedly influenced by the lipid content of the cells. Interestingly though, the half-life of the retinoic acid within the cells is markedly different. From Fig. 3*A*, we estimate the half-life of retinoic acid in the BFC-1β adipocytes to be approximately 1.25 h, whereas the half-life in BFC-1β preadipocytes is approximately 5 h. Because neither BFC-1β preadipocytes nor BFC-1β adipocytes express cellular retinoic acid-binding protein (CRABP) (24), an intracellular retinoid-binding protein which is thought to play a role in the oxidative metabolism of retinoic acid (7), it would appear that metabolism of retinoic acid in these cells in not dependent on CRABP presence and that these two cell types have markedly different capacities for retention and metabolism of retinoic acid.

DISCUSSION

It has long been known that, under normal physiologic and dietary conditions, all-*trans*-retinoic acid is present in the circulation, albeit at very low levels (at 0.2–0.7% of those of

TABLE V

Relative tissue distribution of all-trans-[^{3}H]retinoic acid and [^{14}C]oleic acid given by simultaneous intravenous bolus dose

Tissue	All-*trans*-retinoic acid		Oleic acid	
	C[a]	D[b]	C[a]	D[b]
Brain[c]	0.088 ± 0.004[d]	0.118 ± 0.025	0.008 ± 0.001	0.011 ± 0.003
Liver[e]	0.790 ± 0.150	2.385 ± 0.481	1.100 ± 0.260	1.620 ± 0.437
Kidney[e]	0.136 ± 0.081	0.430 ± 0.177	0.175 ± 0.076	0.330 ± 0.098
Epididymal fat	0.017 ± 0.013	0.045 ± 0.038	0.016 ± 0.009	0.042 ± 0.032
Perinephric fat	0.065 ± 0.041	0.131 ± 0.071	0.073 ± 0.021	0.152 ± 0.080
Seminal vesicle[c]	0.019 ± 0.005	0.024 ± 0.006	0.004 ± 0.001	0.006 ± 0.002
Epididymis[c,e]	0.003 ± 0.001	0.022 ± 0.017	0.002 ± 0.002	0.007 ± 0.005
Pancreas[e]	0.048 ± 0.209	0.206 ± 0.740	0.055 ± 0.010	0.182 ± 0.059
Testis[c]	0.008 ± 0.005	0.012 ± 0.005	0.003 ± 0.001	0.003 ± 0.002

[a] Rats were maintained for 10 weeks on a nutritionally complete, purified control diet containing 2.4 μg of retinol/g of diet.

[b] Rats were maintained for 10 weeks on a totally retinoid-deficient but otherwise nutritionally complete diet.

[c] Tissues which took up significantly (5% level of significance) more all-*trans*-retinoic acid than oleic acid regardless of dietary group.

[d] All values are expressed as percent of injected dose per g of tissue present 10 min after injection. Each value is given as the mean ± S.D. for four animals.

[e] Tissues which took up significantly (5% level of significance) more all-*trans*-retinoic acid when the animal was maintained on the totally retinoid-deficient diet.

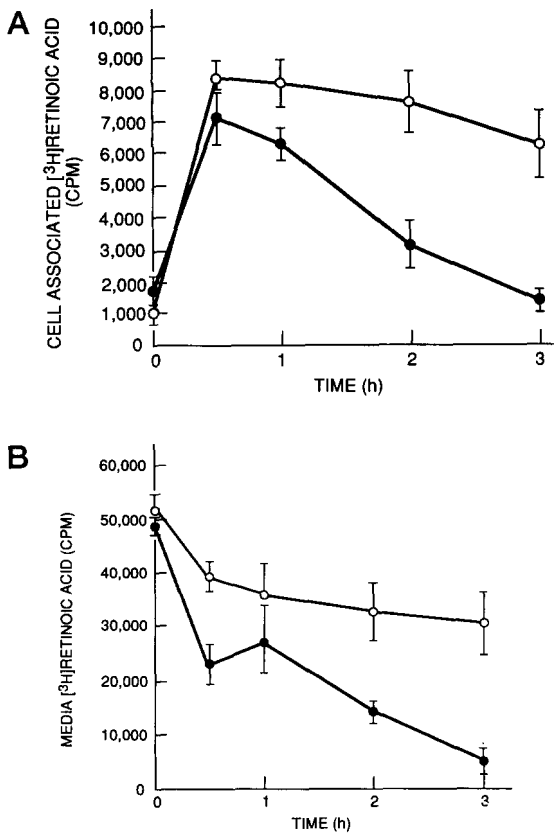

FIG. 3. **Uptake and metabolism of all-*trans*-[^{3}H]retinoic acid by BFC-1β preadipocytes (*open circles*) and adipocytes (*closed circles*).** Confluent cultures of BFC-1β preadipocytes and adipocytes were provided with 0.5 μCi of all-*trans*-[^{3}H]retinoic acid (at a final concentration of 3×10^{-8} M) in the appropriate culture medium at 0 h. At the indicated times, cells (*Panel A*) and medium (*Panel B*) were taken for HPLC analysis of all-*trans*-[^{3}H]retinoic acid as described under "Experimental Procedures."

plasma retinol) (7, 13–16). However, no information is presently available regarding the physiologic role of plasma retinoic acid. The studies reported here were designed to investigate the possible role of circulating all-*trans*-retinoic acid in providing tissues with this retinoid. Our data indicate that, for the brain and liver, greater than three-fourths of the all-*trans*-retinoic acid present in these tissues is derived from the circulation. In marked contrast, less than 1% of the all-*trans*-retinoic acid present in the testis is derived from the circulation. As seen in Table II, most of the 10 tissues we examined took up significant amounts of all-*trans*-retinoic acid from the circulation. Thus, circulating all-*trans*-retinoic acid plays an important role for making this retinoid available to tissues.

The values obtained for the fractional catabolic and absolute catabolic rates for all-*trans*-retinoic acid indicate that this retinoid is rapidly turning over in the whole animal. The data obtained from the continuous infusion studies indicate that plasma pools of all-*trans*-retinoic acid are being turned over every 2 min. For these studies, it is possible that some of the infused all-*trans*-[^{3}H]retinoic acid did not enter the circulation or was metabolized rapidly in a nonspecific fashion. (Any loss of infusate has no effect on plasma contribution calculations.) In fact, however, all-*trans*-[^{3}H]retinoic acid accounted for most of the plasma radioactivity at early time points after the start of infusion. Also, even if 75% of the infusion is lost, the FCR becomes 7.5 instead of 30 pools/h, still indicating very rapid turnover. In the bolus studies, 20–40% of the injected all-*trans*-[^{3}H]retinoic acid was in plasma at 1 min, indicating substantial integrity of the dose. The literature indicates that plasma and tissue retinol turns over in the rat at a much slower rate than retinoic acid (34, 35). If the average tissue concentration of all-*trans*-retinoic acid is taken as 4 ng/g (see Table I), this would mean that 2000 ng of all-*trans*-retinoic acid is present in the entire body of a 500-g rat. Since the ACR for all-*trans*-retinoic acid is 192 ng/h, this indicates that, for control rats, the whole body pool of all-*trans*-retinoic acid is replaced approximately once every 10 h.

Because our studies indicate that most tissues are able to take up all-*trans*-retinoic acid from the circulation, we wanted to gain insight into the physiologic processes and mechanisms responsible for this uptake. In particular, we wanted to understand whether retinoic acid accumulation by tissues is specific and regulatable in response to physiologic state or whether it is nonspecific and not regulated. Since both retinoic acid and fatty acids are transported in the circulation bound to albumin, we first asked whether retinoic acid and oleic acid are cleared from the circulation via common processes. As seen in Fig. 2 and Table IV, plasma oleic acid is cleared more rapidly than plasma retinoic acid. In addition, retinoid nutritional status was found to influence the rate of retinoic acid but not the rate of oleic acid clearance from the plasma. Overall, these data suggest that retinoic acid accumulation by cells and tissues occurs through processes which are tissue and cell type specific and which are responsive to physiologic (*e.g.* nutritional) state.

It seemed possible that uptake of retinoic acid by cells might depend simply on the general ability of the cell to take up and store lipid. Adipose tissue plays an important role in whole body retinyl ester storage (23), expresses nuclear receptors for

retinoic acid (23, 36), and influences the tissue distribution of retinoic acid and its derivatives, through sequestration of the retinoids, when these compounds are given in large pharmacologic doses (37). Together these observations might suggest that the lipid-rich adipose tissue can play an important role in retinoic acid accumulation and storage. To address this possibility, we asked whether the lipid content of a cell influences either the rate of uptake of all-*trans*-retinoic acid by, or half-life within, a cell. Our studies with BFC-1β preadipocytes and adipocytes (Fig. 3) indicate that cellular lipid content does not influence the rate of uptake of all-*trans*-retinoic acid by cells. Considering their high lipid content, BFC-1β adipocytes might have been expected to retain or sequester retinoic acid more readily than cells containing less lipid, but we found that the adipocytes catabolize all-*trans*-retinoic acid far more rapidly than the relatively lipid poor BFC-1β preadipocytes. It would appear for these two cell types that the half-life of retinoic acid within the cell is controlled by mechanisms unrelated to cellular lipid content. In addition, we have shown that all-*trans*-retinoic acid is present both in epididymal and perinephric adipose tissue in concentrations similar to those in most other tissues. Hence, although adipose tissue may take up and harbor large quantities of retinol, retinyl ester and retinoic acid given in very large pharmacological doses to rodents (37), our data indicate that, although adipose tissue represents a large tissue pool for all-*trans*-retinoic acid, it does not seem to be a major storage site of all-*trans*-retinoic acid under normal physiologic circumstances and that the lipid content of a cell does not influence retinoic acid uptake from the circulation.

A striking finding of our studies was that the testis, which possesses all-*trans*-retinoic acid at a level similar to the other tissues (see Table I), derives almost none of this retinoid from the circulation. Since 1925, it has been known that a rat maintained in the total absence of dietary retinol, but supplemented with all-*trans*-retinoic acid in the diet, will be generally healthy, but blind and sterile (1, 38). Because retinaldehyde is the active retinoid in the visual cycle and since all-*trans*-retinoic acid cannot be reduced to retinaldehyde, the inability of dietary supplementation with all-*trans*-retinoic acid to restore vision is understood (39). It is known that the lesion in reproduction arises from a failure of spermatogenesis, which shows an obligatory requirement for retinol in the diet. However, the biochemical basis for the inability of all-*trans*-retinoic acid to substitute for the obligatory retinol needed during spermatogenesis has not been understood. A recent study indicated that when a large dose of all-*trans*-retinoic acid (5 mg) was injected intratesticularly into a retinoid-deficient rat in which spermatogenesis was blocked, spermatogenesis rapidly resumed (40). This suggests that retinoic acid can promote spermatogenesis. Our data indicate that a barrier exists in the testis for the uptake of all-*trans*-retinoic acid from the circulation. This would explain why dietary all-*trans*-retinoic acid cannot substitute for retinol to maintain spermatogenesis. The biochemical nature of the testis barrier to all-*trans*-retinoic acid is not clear. It is known that retinoic acid, at physiologic pH, readily traverses membranes and it is generally thought that retinoic acid enters cells by passive diffusion (41, 42). Thus, it would seem unlikely that a physical barrier in the testis, such as the lack of a cell surface receptor for retinoic acid, could account for the inability of the plasma to contribute to testis all-*trans*-retinoic acid pools. Alternatively, it is possible that a metabolic barrier, consisting of enzymes that rapidly metabolize all-*trans*-retinoic acid, functions to prevent entry of all-*trans*-retinoic acid into certain testicular cells from plasma. To investigate this possibility, we isolated three cell fractions from testes of rats continuously infused to steady state with all-*trans*-[^{3}H]retinoic acid. These cell fractions consisted of a Sertoli + germ cell fraction, an interstitial (Leydig) cell fraction, and a peritubular cell fraction (43). In three independent experiments, 72.5, 59.7, and 39.5% of the total ^{3}H counts/min present in the testes were found in the peritubular cell fraction (data not shown). The remainder of the ^{3}H counts/min were found to be distributed nearly equally between the Sertoli + germ cell fraction, the interstitial cell fraction, and extracellularly in the isolation medium. Based on these preliminary studies, it would seem that the peritubular cells may serve as the cellular site for a metabolic barrier that prevents circulating retinoic acid from entering the interior of the seminiferous tubules where spermatogenesis occurs.

In summary, our studies show that plasma all-*trans*-retinoic acid plays an important role, under normal physiologic conditions, in the delivery of retinoic acid to tissues. They provide an explanation for an observation that is over 65 years old, regarding why dietary all-*trans*-retinoic acid (*i.e.* delivered by the circulation) will not substitute for dietary retinol and maintain spermatogenesis in rats. Studies with preadipocytes and adipocytes indicate that the cellular processes responsible for accumulating all-*trans*-retinoic acid are cell type-specific, and studies of the uptake by tissues of bolus doses of all-*trans*-retinoic acid indicate that these processes are also tissue-specific. Moreover, the uptake of all-*trans*-retinoic acid is responsive to physiologic (*e.g.* nutritional) state. Together, these data indicate that the delivery of all-*trans*-retinoic acid by plasma to tissues is a dynamic and important process for maintaining tissue retinoic acid pools.

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